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ROLE OF DNA POLYMERASE α IN CHROMOSOMAL ABERRATION PRODUCTION
BY IONIZING RADIATION¹

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1. Introduction

Aphidicolin is a tetracyclic diterpinoid fungal antibiotic which inhibits DNA synthesis in eukaryotic cells by interfering specifically with DNA polymerase α , but not polymerases β or γ ¹. It appears that aphidicolin inhibits by binding to and inactivating the DNA-polymerase α complex². We have shown that aphidicolin, like other inhibitors of DNA synthesis, both induces chromosomal aberrations in human peripheral lymphocytes, and, as a post-treatment, interacts synergistically with X rays to produce greatly enhanced aberration yields³. Because DNA polymerase α is the only DNA synthetic or repair enzyme known to be affected by aphidicolin, we infer that this enzyme is directly involved in the repair of DNA lesions which, if unrepaired, can result in visible chromosomal aberrations.

The present experiments were undertaken to further explore the effects of aphidicolin in human lymphocytes in the post-DNA-synthetic G₂ phase of the cell cycle. Earlier experiments in which cells were simply fixed at times after treatment when the frequency of metaphases that are in the DNA-synthetic S phase of the cell cycle is zero in typical percentage labeled mitoses curves for human lymphocytes did not completely rule out the possibility that the aberrations induced by aphidicolin actually arose in a small subpopulation of cells actually in the S phase, and not in G₂ cells. Furthermore, the yield of X-ray-induced aberrations in G₂ cells falls rapidly as a function of increasing irradiation-fixation interval⁴, so comparisons of yields at particular fixation times can be misleading if the cells in each group do not progress through G₂ at the same rate. The experiments here reported utilized labeling with tritiated thymidine to

positively identify cells in the S phase at the time of treatment, and serial colcemid collections and fixations to determine aberration yields over as much of the G₂ phase as feasible.

2. Materials and Methods

2.1 Lymphocyte culture

Sterile, heparinized blood samples were obtained from normal, healthy, adult volunteers. Whole blood was inoculated into 10 ml of RPMI 1640 (GIBCO) containing 15% foetal calf serum in a quantity sufficient to give 5×10^6 leukocytes per culture, based upon prior cell counts. Cultures were incubated at 37°C in 15 ml screw-capped conical plastic centrifuge tubes following addition of 0.25 ml of phytohemagglutinin (GIBCO). They were incubated for 72 hr prior to treatment. One and one-half hours prior to fixation, colcemid was added to a level of 0.1 µg/ml.

2.2 Aphidicolin

The drug was the generous gift of Dr. A.H. Todd of Imperial Chemical Industries, Ltd. It was dissolved in dimethyl sulfoxide to give a 5×10^{-3} M stock solution which was stored at -20°C. It was added to treated cultures to give a final concentration of 5×10^{-5} M. The dimethyl sulfoxide concentration in cultures was thus 1.0%, and all controls also had solvent added to this level. The drug or solvent was added immediately after the X ray treatments and allowed to remain while the culture was reincubated for the indicated treatment times. If the drug or solvent was removed, this was done by aspiration of the supernate from centrifuged cultures, followed by two washes with pre-warmed medium. All procedures were carried out in a warmroom at 37°C to avoid temperature-induced effects on progression through the cell cycle.

2.3 Labeling

Immediately before treatment tritiated thymidine (^3H -Methyl; 2 Ci/mM; Amersham) was added to the cultures to a level of 1 μ Ci/ml in order to tag S phase cells. The label was allowed to remain until drug or solvent was washed out (or until fixation).

2.4 X-irradiation

Cultures were irradiated with a General Electric Maxitron operated at 250 KVp with 1.0 mm Al and 0.5 mm Cu added filtration. The exposure rate was approximately 100 R per minute. Irradiations were done at room temperature, though the cultures were removed from incubation immediately before irradiation and returned immediately after, and so probably did not cool very much during irradiation.

2.5 Experiments

Two types of experiment were done. In the first, an X ray exposure of 100 R was used, and the aphidicolin or solvent remained until the time of fixation. Colcemid was added either immediately after irradiation and/or drug addition, or after 1.5, 3, 4.5 or 6 hr, and the cultures fixed 1.5 hr later at 1.5, 3, 4.5, 6 and 7.5 hr, respectively. Thus mitoses were collected through successive 1.5 hr "windows" giving samples of cells treated at successively earlier stages, with successively longer and longer aphidicolin post-treatment times.

The second type of experiment was carried out according to the same general plan, except that the aphidicolin or solvent was washed out of the cultures fixed at 3 hr and later after 1.5 hr of post-treatment time (e.g. immediately prior to addition of colcemid to the cultures to be fixed at 3 hr). Thus successive 1.5 hr "windows" were again sampled, but this time following a constant 1.5 hr post-treatment immediately following X-irradiation.

2.6 Fixation and slide preparation

Cultures were treated for 15 min in 75 mM KCl, fixed in 3:1 methanol:acetic acid, washed twice with fresh fixative, spread on the surfaces of clean wet slides and air dried. They were then dipped in Kodak NTB emulsion diluted 1:1 with distilled water, dried, and stored for approximately 2 weeks exposure time in light-proof boxes at 4°C. Autoradiographs were developed in D-19 developer (Kodak), fixed, and subsequently stained with 5% Giemsa in phosphate citrate buffer at pH 5.75 for 20-30 min. Metaphases were scored for presence or absence of label, and unlabeled mitoses with 44-46 chromosomes scored for chromosomal aberrations according to conventional criteria.

3. Results

3.1 Continuous post-treatment

Two separate experiments involving a total of three subjects were done. As no significant differences were seen between them, all results are pooled over all three donors. The X ray dose was 100 R. Percentages of labeled mitoses were ascertained on 200 metaphase samples for each treatment of each time point. No labeled mitoses were seen at 1.5 hr; for the solvent control the 50% labeled point was at 3.0 hr, while the X ray-plus-solvent-treated cells reached the 50% point somewhat later at about 4.5 hr. Naturally, neither of the aphidicolin-treated cultures showed any labeled metaphases at any fixation time. Unexpectedly, however, though metaphases were plentiful in cultures treated with aphidicolin alone or with X rays alone, the 3 hr cultures given both X rays and aphidicolin from all three donors contained very few mitoses, and none from any donor contained more than an odd mitoses here and there by 4.5 hrs nor any at all in the later fixations.

The pooled results for aberrations at 1.5 hr scored in 150 metaphases per donor per point are shown in Table 1, and are typical of our earlier results³. Only chromatid aberrations, mainly achromatic lesions and chromatid deletions, were seen. Their frequency was significantly, if not dramatically, increased by aphidicolin alone. The yields for the combined treatment was greater than the sum of the yields for either X rays or aphidicolin alone; 2.5 times in the case of achromatic lesions and over 4 times in the case of chromatid deletions. Just as in our earlier experiments, though X ray alone induced some chromatid exchanges, none at all were seen in aphidicolin-treated cultures.

Because of the paucity of mitoses in the X ray-plus-aphidicolin cultures it was possible to score only a total of 170 mitoses on the slides from all three donors. Scoring of mitoses from the aphidicolin alone and the X rays alone samples was accordingly limited to an arbitrary total of 250 cells distributed among donors approximately as were the metaphases from the combined treatment. Despite the smaller samples, the pooled data for the 3 hr fixation still shows a dramatic effect. As expected, the frequencies of aberrations in the cultures treated with X rays alone are substantially lower than for the first collection interval, while those in the cultures treated with aphidicolin alone are significantly increased. The excesses over additivity for the combined treatment are five-fold for achromatic lesions and nine-fold for deletions, or about twice as much as in the 1.5 hr samples.

3.2 Limited post-treatment

Only one experiment involving blood samples from two different donors has been done. This time, to try to minimize induced delay, an X ray dose of 50 R was used. Again, because there were no significant differences in

the results for the two donors they have been pooled for presentation here. Figure 1 shows the percentages of labeled mitoses in graphic form. As expected, removal of the aphidicolin did allow labeled mitoses to appear eventually, though it will be seen that the 50% labeled mitoses point is still reached at successively later sampling intervals according to treatment; in the solvent control cultures again by about 3 hr, in the X-irradiated perhaps an hour later at about 4 hr, and in the aphidicolin-treated by about 6 hr, while the cultures receiving the combined treatment never achieved more than 30% labeling of their mitoses.

The pooled results for chromosomal aberrations are shown in Table 2. An attempt was made to score 100 metaphases per donor per point, but as may be seen this was not always achieved, particularly for the later times. This was caused by a paucity of mitoses in general and particularly of the required unlabeled mitoses for the less drastic treatments in later collection intervals. Despite the lower X ray exposure, the combined treatment still resulted in a substantial increase in chromatid deletions over additivity at 1.5 hr, though not much effect is any longer apparent for achromatic lesions.

Aberration frequencies as a function of sampling time are shown graphically in Fig. 2. As is commonly seen, the samples treated with X rays alone show a rapid decrease with time in aberration frequencies, a decrease approximating an exponential decay curve. Clearly, however, the situation is more complex with the combined treatment. It is immediately apparent that the synergistic effect for chromatid deletions has completely disappeared after the first 1.5 hr collection interval; indeed it appears significantly reversed in the 3.0 and 4.5 hr samples. In somewhat similar

fashion, achromatic lesion frequencies, comparable in the 1.5 hr samples, are significantly depressed in the 3.0 and 4.5 hr samples. Interestingly, the trend for both aberration categories is toward increased yield for the combined treatment in the latest collection intervals. Had we sampled even later, we might have seen a return to a synergistic effect as is suggested by the curves drawn through the data points in Fig. 2.

4. Discussion

These experiments confirm our earlier observations³ that aphidicolin induces aberrations in G₂ lymphocytes, that post-treatment with aphidicolin results in a large synergistic increase in the production of chromosomal aberrations in lymphocytes X-irradiated during the latter portion of the G₂ phase of the cell cycle, and that exchanges are not formed in the presence of aphidicolin. Dr. R.C. Moore and I have recently determined the effect of various concentrations of aphidicolin on normal "semiconservative" DNA synthesis in human lymphocytes and in several experiments found it to decrease exponentially (as measured by scintillation counting after exposure of the cells to tritiated thymidine) over several decades of concentration, with minimal effect at about 5×10^{-6} molar and 99% inhibition at about 10^{-3} molar (unpublished data). At the concentration used in the present experiments, DNA synthesis is about 80% inhibited as measured this way. We have found in addition (also unpublished) that both aberration induction by aphidicolin alone and the synergistic increase in aberration induction by X rays is a linear function of aphidicolin concentration over the same range. As we speculated earlier, the conclusion that these effects result from inhibition of some sort of DNA repair process seems inescapable. Though inhibition of more than one enzyme cannot be ruled out, it seems not

unlikely that polymerase α functions as a repair enzyme as well as in normal DNA synthesis. Because only unlabeled mitoses were scored in the present experiments, the cells in which aberrations were induced by aphidicolin alone had, insofar as it is possible to determine, completed their S phase. Thus the mechanism seems most likely to involve inhibition of either some "final assembly" or some "maintenance" function in which too few bases are inserted to be detected autoradiographically.

The rationale of the present experiments lies in the need to determine aberration yields in G_2 cells over a sufficiently long sampling interval to ensure that most of the cells actually in G_2 in the treated population are included in the sample before concluding that two treatments (or two types of cell) generate different responses. This is of course because shifts in the normal progression of cells to metaphase can generate large apparent differences in yield as a consequence of sampling of cells actually in different G_2 substages, possibly of differing sensitivity, even though arriving at metaphase simultaneously. What is needed, then, amounts to a comparison of the integral of aberration yields over all G_2 cells. Furthermore, in simple cases where aberration yields are consistently different for all sampling intervals and curve shapes are the same, it is possible to ask whether the two curves are different in slope, in intercept, or both. Such information can provide insight into mechanism; for example a difference in intercept might imply a different number of lesions induced initially, while a difference in slope might imply that lesions are repaired at different rates. We have recently used this approach successfully to explore the G_2 chromosomal radiosensitivity of lymphocytes from persons affected with ataxia telangiectasia⁵.

The apparent failure of a limited aphidicolin post-treatment to interact synergetically with X-irradiation in metaphases collected over intervals beyond that during which it is actually present, as in the experiment shown in Table 2 and Fig. 2, is difficult to interpret. As a single experiment, it will naturally have to be repeated before final conclusions are drawn. Nevertheless, the data from the two donors were consistent, and those for successive collection intervals appear to conform well to smooth curves, and thus seem unlikely to represent a simply capricious result. A more significant problem lies in the fact that the percentages of labeled mitoses in samples of aphidicolin-treated cells never reached the high levels that would suggest that most of the G_2 cells had been sampled (Fig. 1). For cells treated with aphidicolin alone this does not appear to constitute a real problem, as aberration yields are low and do not appear to change in any consistent way from the earliest to the latest collection interval. However, for the combined treatment not only did the percentage of labeled cells never exceed about 30%, implying that the experiment may have failed to sample a large cohort of G_2 cells at all, but the aberration yield appears to be increasing again during the last two collection intervals after reaching a nadir during the 3.0 - 4.5 hr interval.

Integrating crudely by simply adding up all the aberration yields for all sampling periods from Table 2, there is no difference between the overall yields of aberrations in the samples given X rays alone and those given X rays plus aphidicolin (for total aberrations the frequencies of aberrations per cell are 0.47 and 0.46, respectively). It might thus be concluded that aphidicolin is not really increasing the yield of X-ray-induced aberrations at all, but rather somehow causing those with aberrations to

reach metaphase earlier. On the other hand, in the light of the important role of DNA repair systems in the production of chromosomal aberrations^{5,6}, it is difficult to discard the idea that the effect is a real one influencing the efficacy with which radiation-induced damage to DNA is repaired. Nevertheless, a definite conclusion must await additional data, particularly for later collection intervals for the combined treatment.

The failure of X-irradiated cells treated continuously with aphidicolin to continue to arrive at metaphase after the first few hours as seen in the experiments summarized in Table 1 strongly suggests that the cells irradiated early in G₂ are for some reason prevented from progression through the cell cycle in the absence of polymerase α function. This is clearly not true, or at least not to the same extent, without the prior X-irradiation, so whatever block there is would seem to involve unrepaired X ray damage.

5. Acknowledgment

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Figure Legends

Figure 1. Percentage labeled mitosis curves for G_2 human lymphocytes treated with 5×10^{-5} M aphidicolin for 1.5 hr, 50 R of X rays, or 50 R of X rays followed by 5×10^{-5} M aphidicolin for 1.5 hr and collected over successive 1.5 hr intervals. See text for details. Closed circles, solvent control; triangles, X ray alone; open circles, aphidicolin alone; X's, X ray plus aphidicolin.

Figure 2. Frequencies of chromosomal aberrations in metaphases of G_2 human lymphocytes treated with 5×10^{-5} M aphidicolin for 1.5 hr, 50 R of X rays, or 50 R of X rays followed by 5×10^{-5} M aphidicolin for 1.5 hr and collected over successive 1.5 hr intervals. See text for details. Hatched area, mean plus and minus Poisson error for cells treated with aphidicolin alone; closed circles, individual aphidicolin values; X's, X rays alone; triangles, X rays plus aphidicolin. Panel A, chromatid aberrations except achromatic lesions; panel B, achromatic lesions.

Figure 1

Figure 2

Table 1. Aberration frequencies (per cell) in human lymphocytes given 100 R of X rays followed by post-treatment with 5×10^{-5} M aphidicolin.

<u>Fixation (Posttreatment) Time</u>	<u>Treatment</u>	<u>Cells Scored</u>	<u>Achromatic Lesions</u>	<u>Chromatid Deletions</u>	<u>Isochromatid Deletions</u>	<u>Chromatid Exchanges</u>
1.5 hr	Control	450	0.05 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0
	Aphidicolin	450	0.13 ± 0.02	0.09 ± 0.01	0.01 ± 0.01	0
	X-rays	450	1.18 ± 0.05	0.95 ± 0.05	0.05 ± 0.01	0.09 ± 0.01
	X-rays + Aphidicolin	450	3.30 ± 0.09	4.43 ± 0.10	0.12 ± 0.02	0
3.0 hr	Aphidicolin	250	0.33 ± 0.04	0.38 ± 0.04	0.06 ± 0.02	0
	X-rays	250	0.26 ± 0.03	0.16 ± 0.03	0.01 ± 0.01	0.01 ± 0.01
	X-rays + Aphidicolin	170	2.94 ± 0.13	4.89 ± 0.17	0.14 ± 0.03	0

Table 2. Aberration frequencies in human lymphocytes given 50 R of X rays followed by a 1.5 hr post-treatment with 5×10^{-5} M aphidicolin or solvent (DMSO) alone and fixed at 1.5 hr intervals.

<u>Fixation Time (hr)</u>	<u>Treatment</u>	<u>Cells Scored</u>	<u>Achromatic Lesions</u>	<u>Chromatid Deletions</u>	<u>Isochromatid Deletions</u>	<u>Chromatid Exchanges</u>	<u>% Labeled Mitoses</u>
1.5	Solvent Control	200	8	1	0	0	16.5
	Aphidicolin	200	42	4	2	0	4.0
	X-rays	161	85	57	7	1	9.0
	X-rays and Aphidicolin	185	88	142	24	2	2.0
3.0	Solvent Control	200	9	3	1	0	53.5
	Aphidicolin	200	30	6	3	0	9.5
	X-rays	170	55	18	14	0	47.5
	X-rays and Aphidicolin	200	31	16	2	0	7.5

Table 2. (Continued)

<u>Fixation Time (hr)</u>	<u>Treatment</u>	<u>Cells Scored</u>	<u>Achromatic Lesions</u>	<u>Chromatid Deletions</u>	<u>Isochromatid Deletions</u>	<u>Chromatid Exchanges</u>	<u>% Labeled Mitoses</u>
4.5	Solvent Control	124	2	1	0	0	85.0
	Aphidicolin	161	19	5	0	0	34.5
	X-rays	200	36	18	7	2	52.5
	X-rays and Aphidicolin	200	23	5	3	0	14.5
6.0	Solvent Control	100	6	2	0	0	99.0
	Aphidicolin	106	12	1	1	0	52.0
	X-rays	140	20	9	6	2	83.0
	X-ray and Aphidicolin	200	30	8	4	1	28.0

Table 2. (Continued)

<u>Fixation Time (hr)</u>	<u>Treatment</u>	<u>Cells Scored</u>	<u>Achromatic Lesions</u>	<u>Chromatid Deletions</u>	<u>Isochromatid Deletions</u>	<u>Chromatid Exchanges</u>	<u>% Labeled Mitoses</u>
7.5	Solvent Control	20	0	0	0	1	99.0
	Aphidicolin	53	3	1	0	0	59.5
	X-rays	109	16	9	0	1	91.5
	X-rays and Aphidicolin	200	58	9	8	2	29.0